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The chromatin remodelling complex WSTF–SNF2h interacts with nuclear myosin 1 and has a role in RNA polymerase I transcription

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Nuclear actin and myosin 1 (NM1) are key regulators of gene transcription. Here, we show by biochemical fractionation of nuclear extracts, protein–protein interaction studies and chromatin immunoprecipitation assays that NM1 is part of a multiprotein complex that contains WICH, a chromatin remodelling complex containing WSTF (Williams syndrome transcription factor) and SNF2h. NM1, WSTF and SNF2h were found to be associated with RNA polymerase I (Pol I) and ribosomal RNA genes (rDNA). RNA interference-mediated knockdown of NM1 and WSTF reduced pre-rRNA synthesis *in vivo*, and antibodies to WSTF inhibited Pol I transcription on pre-assembled chromatin templates but not on naked DNA. The results indicate that NM1 cooperates with WICH to facilitate transcription on chromatin.

Keywords: nuclear myosin 1; WSTF; SNF2h; chromatin remodelling complexes; rDNA; Pol I transcription

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INTRODUCTION

Dynamic transitions of chromatin structure induced by ATP-dependent chromatin remodelling complexes have been shown to either facilitate or prevent the access of the transcription machinery to gene promoters (Lusser & Kadonaga, 2003; Flaus & Owen-Hughes, 2004). Moreover, transcription elongation through chromatin templates is driven by ATP-dependent remodelling complexes that modify chromatin and mobilize nucleosomes (Belotserkovskaya & Reinberg, 2004). In mammals, there are at least four classes of chromatin remodelling complexes, containing the ATPases SWI2/SNF2, Mi-2, ISWI/SNF2 or INO80 (Sif, 2004; Mohrmann & Verrijzer, 2005). These remodelling complexes act as molecular machines that exert essential functions both in the assembly and maintenance of chromatin structure, as well as DNA replication and transcription regulation (Corona & Tamkun, 2004; Sif, 2004). The function of the different remodelling complexes in specific chromatin-based processes has just begun to be explained. For example, NoRC, a complex consisting of TIP5 and SNF2h, associates with the promoter of silent ribosomal RNA genes and represses polymerase I (Pol I) transcription by recruiting histone-modifying enzymes and DNA methyltransferase to rDNA (Strohner *et al*, 2001; Santoro *et al*, 2002). Another member of remodelling complexes, termed WICH, consisting of SNF2h and WSTF (Williams syndrome transcription factor) is recruited to replication foci and prevents aberrant heterochromatin formation shortly after DNA replication, thereby allowing rebinding of factors to newly replicated DNA (Bozhenok *et al*, 2002). WICH is targeted to replication foci through the binding of WSTF to the DNA clamp protein proliferating-cell nuclear antigen (Poot *et al*, 2004), suggesting that this complex is required for maintaining the chromatin structure during replication. Besides this, WSTF acts as a component of the multisubunit SWI/SNF complex WINAC as regulator of vitamin D-coupled transcription (Kitagawa *et al*, 2003; Fujiki *et al*, 2005).

Recent studies have shown that actin and myosin are found in the nucleus and are required for transcription by all three classes of nuclear RNA polymerases (Pestic-Dragovich *et al*, 2000; Fomproix & Percipalle, 2004; Hofmann *et al*, 2004; Hu *et al*, 2004; Philimonenko *et al*, 2004; Kukalev *et al*, 2005; Visa, 2005). Actin works together with nuclear myosin 1 (NM1) to drive Pol I transcription; the manner in which it does this, however, is not yet

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known. Here, we present new insights into the function of NM1 in rDNA transcription, showing that NM1 is required not only for transcription initiation but also for a post-initiation step, such as promoter escape or elongation. It is noteworthy that NM1 is part of a large multiprotein complex containing WSTF and SNF2h. The complex colocalizes with Pol I in nucleoli and interacts with Pol I, and RNA interference-mediated knockdown of WSTF impairs pre-rRNA synthesis. The results indicate that a 2–3 MDa multiprotein complex containing NM1, WSTF and SNF2h is required for rDNA transcription.

RESULTS AND DISCUSSION

In situ colocalization of NM1 and Pol I

Immunostaining of isolated nucleoli showed that actin and NM1 were enriched in nucleoli and their distribution correlated with upstream binding factor (UBF) and fibrillarin (Fig 1A). NMI colocalized with Pol I and green fluorescent protein–RPA53, a protein that decorates the active subpopulation of Pol I (Fig 1B). The colocalization between Pol I and NM1 was further corroborated by immunogold electron microscopy using intact HeLa cells (supplementary Fig 1 online). NM1 was distributed throughout the nucleoplasm but was largely excluded from the nucleoli, except the fibrillar centres. These nucleolar subcompartments are known to contain Pol I and Pol I-specific transcription factors (Scheer & Benavente, 1990; Dundr *et al*, 2002). In addition, both a novel autoimmune serum against Pol I (S57299; see supplementary Figs 2,3 online) and a peptide-specific affinity-purified antibody against NM1 blocked Pol I transcription *in vitro* (Fig 1C,D). These data support previous studies demonstrating a key role for NM1 in Pol I transcription (Fomproix & Percipalle, 2004; Philimonenko *et al*, 2004).

NM1 is associated with WSTF and SNF2h

Recently, WSTF and SNF2h were found to be contained in a large multiprotein complex, termed B-WICH (Cavellán *et al*, unpublished observations). As NM1 was found in this complex, we wondered whether NM1 was physically associated with B-WICH. To test this, we fractionated nuclear proteins by chromatography on phosphocellulose, MonoQ and Superose 6HR and monitored co-purification of NM1 on immunoblots (Fig 2A). Cellular NM1 was found to co-elute with WSTF and SNF2h in fractions containing protein assemblies with an apparent molecular mass of 2–3 MDa. This indicates that NM1 is associated with a fraction of WICH, a remodelling complex that has a role in the replication of heterochromatin (Bozhenok *et al*, 2002; Poot *et al*, 2004). Significantly, NM1 did not co-elute with NoRC, a complex containing TIP5 and SNF2h that has a role in heterochromatin formation and silencing of the rDNA locus (Strohner *et al*, 2001). To demonstrate the association of NM1 with the 2–3 MDa WSTF–SNF2h complex, fractions from the Superose 6HR column were incubated with anti-WSTF antibodies, and co-precipitated NM1 was monitored on immunoblots. The results in Fig 2B show that NM1 co-precipitated with WSTF from fractions containing 2–3 MDa but not ~1 MDa complexes. Conversely, SNF2h was co-precipitated from both fractions (Fig 2B). A stronger association of NM1 with the WSTF–SNF2h complex was observed in nuclear extracts (Fig 2C,D), consistent with NM1 associating with WICH in a dynamic fashion, and high protein concentrations stabilizing the interaction of NM1 with WSTF. Quantification of proteins

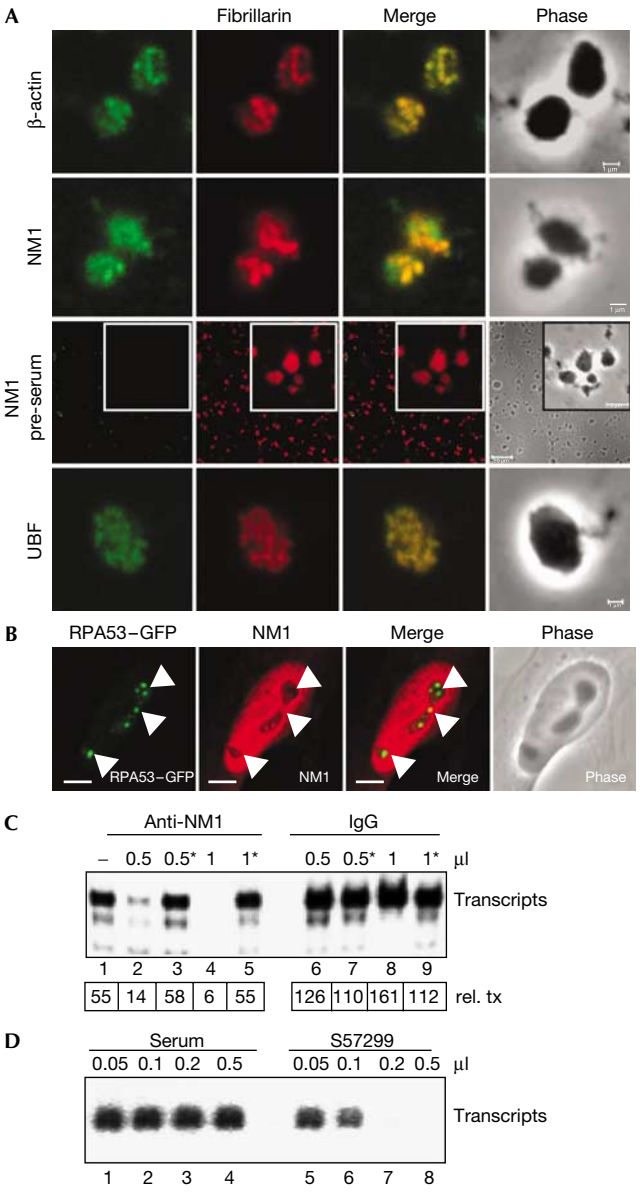

Fig 1 | NM1 localizes in nucleoli and is required for Pol I transcription. (**A**) Confocal sections of isolated nucleoli immunostained with antibodies to β-actin, NM1, UBF and fibrillarin (bar, 1 µm), as well as an anti-NM1 pre-immune serum (NM1 pre-serum; scale bar, 20 or 5 µm in the inset; supplementary information online). (**B**) Immunostaining and confocal microscopy showing colocalization of NM1 with green fluorescent protein (GFP)–RPA53 overexpressed in HeLa cells (scale bar, 5 µm). (**C**) Anti-NM1 antibodies inhibit Pol I transcription *in vitro*. A 10 µg fraction of a DEAE-280 fraction was pre-incubated for 30 min at 30 °C with 0.5 or 1 µl of native or heat-treated (marked by asterisks) anti-NM1 antibodies (lanes 1–4) or rabbit IgGs (lanes 5–8) before transcription was started. The numbers below show the quantification of the transcriptional activities. (**D**) The Pol I-specific autoimmune serum S57299 inhibits Pol I transcription. HeLa nuclear extracts were pre-incubated for 20 min with increasing amounts of a control human serum (lanes 1–4) or S57299 (lanes 5–8) before transcriptional activity was assayed in the presence of template $pHrP_2$ linearized with *Eco*RI. A western blot showing that S57299 recognizes the largest subunit of Pol I (RPA194) is shown in Fig 3B.

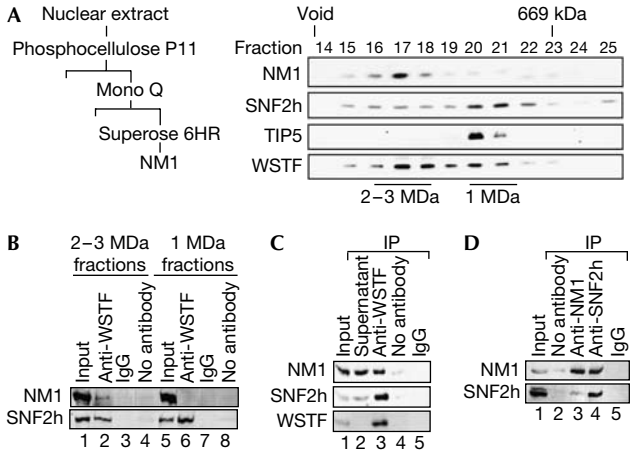

Fig 2 | NM1 is associated with WSTF–SNF2h. (**A**) Resolution of NM1-containing complexes by gel filtration. Proteins were fractionated according to the scheme on the left. Fractions of the Superose 6HR column were analysed on western blots. The position of the 670 kDa marker (thyroglobulin) is indicated. (**B**) Co-precipitation of NM1 with anti-WSTF antibodies from Superose 6HR fractions containing ~2–3 MDa but not ~1 MDa protein complexes. In lanes 1 and 4, 20% of the input material was loaded. (**C**) A complex containing NM1, WSTF and SNF2h is precipitated from nuclear extracts with anti-WSTF antibodies. 10% of the input is shown in lane 1. IP, immunoprecipitation. (**D**) Co-precipitation of NM1 and SNF2h from nuclear extracts. 10% of the input is shown in lane 1.

precipitated with anti-WSTF antibodies shows that about 10% of cellular NM1 is associated with WSTF. These results support the view that NM1, WSTF and SNF2h are physically associated and are contained in a large multiprotein complex.

The NMI–WSTF–SNF2h complex binds Pol I and rDNA

The finding that NM1 is associated with Pol I and B-WICH suggests that this remodelling complex serves a nucleolar function. In support of this, WSTF, SNF2h and NM1 are enriched in isolated nucleoli (Fig 3A), suggesting that the fraction of WICH that contains NMI may be involved in Pol I transcription. To analyse whether both NM1 and WICH are associated with Pol I, a human autoimmune serum (S57299) that recognizes the largest subunit of Pol I (RPA194) was used to immunoprecipitate Pol I from untreated cells or cells that have been crosslinked with the cell-permeable, thiol-cleavable agent dithiobis-succinimidyl-propionate (DSP). The blots in Fig 3B show co-precipitation of NM1, SNF2h, WSTF and actin with Pol I from nuclear extracts of DSP-treated cells, even when nuclear extracts were prepared in the presence of 8 M urea (supplementary information online). In the absence of the crosslinker, however, no significant levels of NMI, WSTF and SNF2h co-precipitated with Pol I. The fact that co-precipitation of the WSTF/SNF2h complex and NMI with Pol I requires *in vivo* crosslinking of proteins indicates that the association with Pol I is relatively weak or very dynamic.

To examine whether WICH is associated with rDNA, chromatin immunoprecipitation (ChIP) experiments were carried out. Previous studies have shown that actin and NM1 are associated with the rDNA promoter (Philimonenko *et al*, 2004), and a proteomic approach has demonstrated the presence of myosin and WSTF in nucleoli (Andersen *et al*, 2005). As shown in Fig 3C, WSTF, SNF2h and NM1 were present at rDNA, occupying both the promoter and the coding region. In contrast, the promoter selectivity factor TIF-IB/SL1 (monitored by antibodies to TAF_I110) was associated with the rDNA promoter but not with the coding region. As a control, an antibody against TFIIIC220 precipitated $tRNA_{lys}$ genes but not rDNA. None of the antibodies precipitated a protein-coding gene (ARPP P0), demonstrating the specificity of the ChIP assays. The finding that NM1 is associated with both the rDNA promoter and the coding region is in apparent contrast to a recent study in which NM1 was found only at the rDNA promoter (Philimonenko *et al*, 2004). This is due to the different anti-NM1 antibodies and ChIP protocols used. Whereas Philimonenko *et al* (2004) have used a bead-bound monoclonal antibody that recognizes the amino terminus of NM1, in this study and in previous work a more sensitive anti-NM1 antibody has been applied (Fomproix & Percipalle, 2004). The different specificity of the NMI antibodies used suggests that NM1 in the transcription initiation complex has a conformation that is different from NM1 that is associated with elongating Pol I.

NM1–WSTF–SNF2h has a role after transcription initiation

The association of NM1, WSTF and SNF2h with both the rDNA promoter and the coding region suggests that these proteins have a role in a post-initiation step of Pol I transcription. To uncouple transcription initiation from elongation, we performed abortive transcription-initiation assays that make use of the fact that Pol I can initiate transcription and cycle short transcripts in the presence of two or three nucleotides that correspond to the 5′-terminal sequence of the respective transcript. In the experiment in Fig 4A, fractionated nuclear extract (DEAE-280 fraction) was incubated with antibodies against NM1, WSTF, Pol I or rabbit IgGs before being supplemented with the rDNA template and ATP/CTP, the first two nucleotides of mouse pre-rRNA. Half the reaction was supplemented with GTP and UTP to allow transcription elongation, whereas the second half was substituted with UTP to synthesize ACU trimers. As expected, antibodies against Pol I inhibited both run-off transcription and the formation of ACU trimers. Conversely, anti-NM1 antibodies inhibited the synthesis of run-off Pol I transcripts without affecting the formation of ACU trimers, and antibodies against WSTF did not affect the synthesis of either run-off or abortive transcripts (Fig 4A, lanes 6,7).

To examine whether WSTF is required for Pol I transcription on chromatin templates, we compared the effect of WSTF antibodies on circular rDNA and on reconstituted chromatin. The template used was $pMrT_2$, a minigene containing rDNA promoter and terminator sequences. As shown in Fig 4B, long read-through transcripts were synthesized in the absence of TTF-I (lane 1), whereas specifically terminated transcripts were produced in the presence of TTF-I (lanes 2–5). As expected, antibodies to WSTF did not affect the synthesis of terminated transcripts on naked DNA (lanes 4,5). A different result was obtained on chromatin templates. Consistent with previous studies (Längst *et al*, 1997), specific transcription on pre-assembled chromatin templates was repressed in the absence of TTF-I. The low level of read-through transcripts in the absence of TTF-I is due to traces of DNA that has not properly assembled into chromatin (lane 6). In the presence of

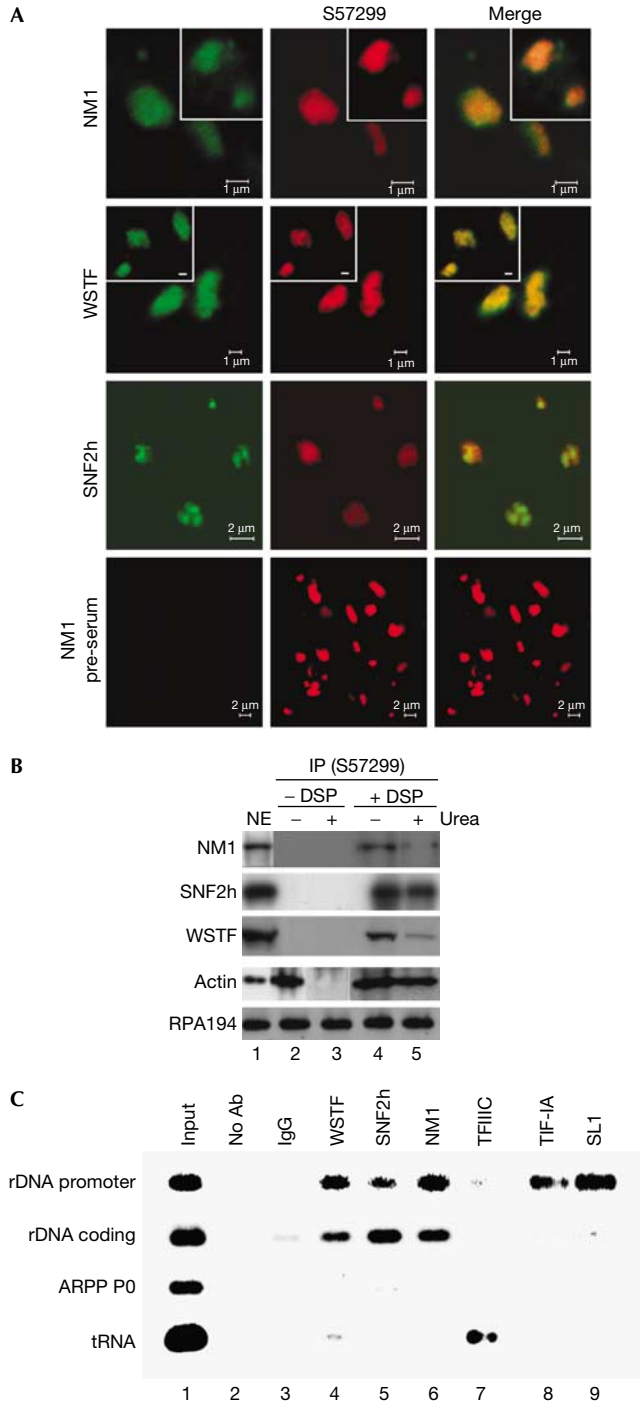

Fig 3 | NM1, WSTF and SNF2h are associated with rDNA and promote 45S pre-rRNA synthesis. (**A**) Nucleolar colocalization of NM1 (scale bar, 1 μm), WSTF (scale bar, 1 μm) and SNF2h with Pol I (scale bar, 2 μm). (**B**) NM1, WSTF and SNF2h are associated with the transcription-competent subpopulation of Pol I. Proteins were crosslinked *in vivo* with dithiobis-succinimidyl-propionate (DSP) and Pol I was precipitated from cell lysates with anti-Pol I antiserum S57299. Co-precipitated NM1, WSTF, SNF2h, actin and RPA194 were detected on immunoblots. IP, immunoprecipitation. (**C**) Chromatin immunoprecipitation analysis. Lysates of crosslinked cells were incubated with the indicated antibodies and co-precipitated DNA was subjected to PCR using primers that amplify the rDNA promoter, the 18S rRNA coding region, ARPP P0 and tRNA genes. Lane 1 shows PCR amplification of 0.5% input chromatin (supplementary information online).

TTF-I, chromatin-mediated transcriptional repression was overcome, and specifically initiated and terminated transcripts were synthesized (lane 7). Significantly, antibodies against WSTF inhibited transcription by Pol I without affecting the synthesis of nonspecific transcripts (lanes 8–10). The finding that WSTF antibodies inhibit Pol I transcription on chromatin templates but not on naked DNA suggests that the WSTF–SNF2h complex facilitates Pol I transcription on chromatin.

If WSTF/SNF2h-mediated chromatin remodelling is required for Pol I transcription, the level of pre-rRNA should be decreased after depletion of WSTF by RNAi-mediated gene silencing. In the experiment in Fig 4C, HeLa cells were transfected with pSUPER-shWSTF, a vector that transcribes WSTF-shRNA as a stem–loop precursor from a Pol III promoter (Brummelkamp *et al*, 2002). After 2 days, the level of WSTF dropped to 50% compared with the empty vector or a respective control plasmid (supplementary Fig 4A,B online). Analysis of WSTF messenger RNA, 18S, 28S and 45S pre-rRNAs, transfer RNA and interleukin-6 (IL-6) mRNA by quantitative reverse transcription–polymerase chain reaction demonstrates that WSTF mRNA decreased to 49% and 45S pre-rRNA to 39% in WSTF-depleted cells compared with control cells, whereas the amount of 18S and 28S rRNAs, tRNAs and IL-6 mRNA did not change after WSTF silencing. Longer incubation of cells expressing WSTF-shRNA led to cell death. This observation, together with the decrease of 45S pre-rRNA after knockdown of WSTF mRNA, underscores the functional significance of WSTF-containing remodelling complexes in Pol I transcription and cell growth.

In summary, these results not only extend previous studies demonstrating a role for actin and myosin in rDNA transcription but provide evidence that nucleolar NM1 acts together with WSTF, a subunit of the chromatin remodelling complexes WICH and WINAC (Bozhenok *et al*, 2002; Kitagawa *et al*, 2003; Poot *et al*, 2004; Fujiki *et al*, 2005). Purification of cellular NM1 showed that a fraction of NM1 is associated with a 2–3 MDa complex, termed B-WICH, which contains WSTF, SNF2h and proteins involved in transcription and processing of rRNA, such as RNA helicase II/Guα, the myb-binding protein 1a and CSB (Cockney syndrome protein B). B-WICH contains pre-rRNA, 5S and 7SL RNA, and assembly of the B-WICH complex requires continuous Pol I and Pol III transcription (Cavellán *et al*, unpublished observations). This suggests that NM1 may recruit WICH to active rDNA repeats (and Pol III genes) to establish a chromatin structure that is required for efficient transcription.

We propose that the molecular mechanisms that establish the transcriptionally active or silent state at the rDNA locus are similar. Previous results have shown that silencing of rDNA is mediated by recruitment of an SNF2h-containing chromatin remodelling complex, NoRC, to rDNA by interaction with the transcription termination factor TTF-1 bound upstream of the rDNA promoter (Strohner *et al*, 2001; Santoro *et al*, 2002). NoRC silences transcription by establishing heterochromatic features that

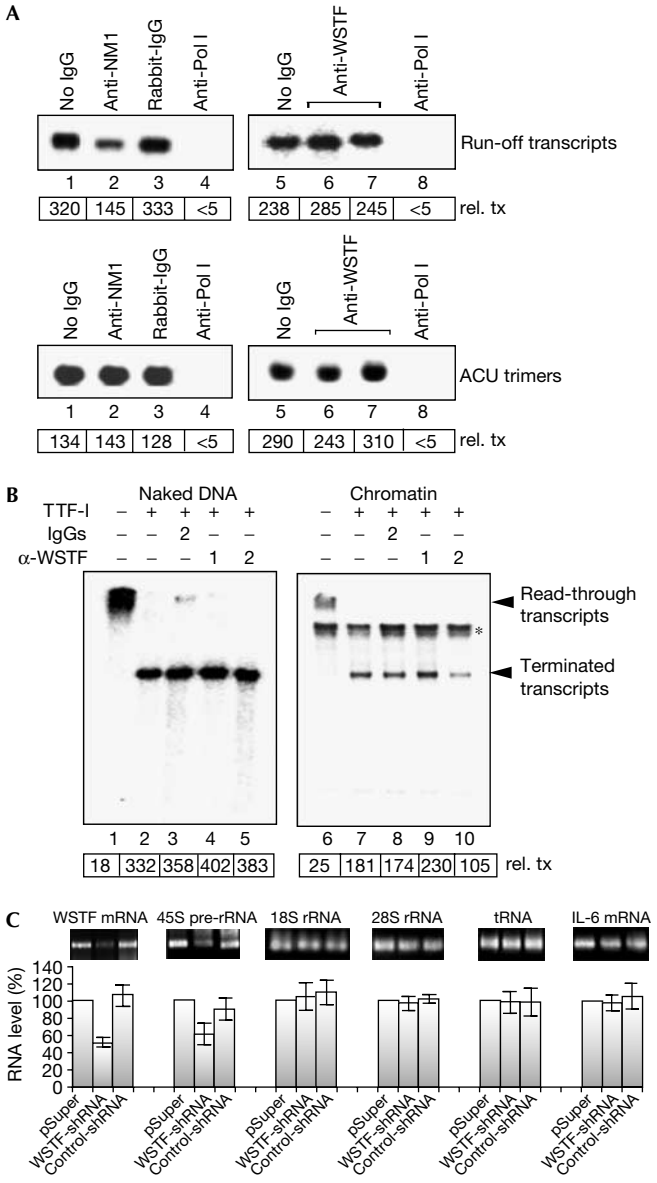

Fig 4 | Both NM1 and the WSTF complex stimulate Pol I transcription. (**A**) Antibodies against NM1 inhibit run-off but not abortive Pol I transcription. A 20 μg portion of fractionated nuclear extract (DEAE-280 fraction) was pre-incubated for 45 min with rabbit IgGs, antibodies to NM1 or WSTF (2 μg each), or 0.1 μl of the anti-Pol I serum S57299 before being assayed for run-off transcription (upper panel) and the synthesis of ACU trimers (lower panel). (**B**) Anti-WSTF antibodies impair rDNA transcription on pre-assembled chromatin templates. Transcription assays contained circular $pMrT_2$ as naked DNA (lanes 1–5) or pre-assembled into chromatin (lanes 6–10). The DEAE-280 fraction was pre-incubated with rabbit IgGs (2 μg) or anti-WSTF antibodies (1 and 2 μg) before transcription was started by adding nucleotides, circular template DNA and recombinant TTF-I (10 ng) as indicated. Read-through and terminated transcripts are indicated; the asterisk marks unspecific transcripts that originate from traces of contaminating DNA in the *Drosophila* extract used for chromatin assembly. (**C**) RNA interference-mediated depletion of WSTF inhibits pre-rRNA synthesis. Cells were transfected with the empty vector ('pSuper'), pSUPER-shWSTF ('WSTF-shRNA') or an unrelated shRNA ('control-shRNA'), and the level of the indicated RNAs was determined by reverse transcription–PCR. A representative experiment is shown on the top. The bar diagram below shows the relative amount of the indicated RNAs determined in five independent experiments. Error bars depict standard deviations estimated by Student's *t*-test. The significance for the WSTF RNAi knockdown is $P = 0.001$ and that for 45S pre-rRNA is $P = 0.032$.

are incompatible with transcription complex formation. B-WICH, on the other hand, may be recruited to active genes by interaction with NMI that is associated with TIF-IA, a factor that mediates the interaction of Pol I with promoter-bound TIF-IB/SL1 (Grummt, 2003). Given that NM1 exerts a positive function in transcription, the interaction of NM1 with WSTF is suggested to recruit the WICH and WINAC complex to active genes to modify chromatin and activate transcription. In support of this, WSTF in the WINAC complex strongly binds acetylated lysines on histones H2B (K12), H3 (K14) and H4 (K16) in genes regulated by vitamin D (Fujiki *et al*, 2005).

METHODS

Immunofluorescence. Isolation of nucleoli, immunostaining and confocal microscopy were carried out as described (Fomproix & Percipalle, 2004). Confocal images were taken from 1 μm sections with an LSM 510 (Zeiss) or Leica TCS-SP Laser Scanning Microscope.

Purification of cellular NM1–WICH complexes. NM1-containing protein complexes were purified from nuclear extracts from HeLa cells by chromatography on Phosphocellulose P-11 (eluted at 0.5–0.65 M KCl) and MonoQ (eluted at 0.35 M KCl) followed by size-exclusion chromatography on Superose 6HR.

Immunoprecipitation and ChIP assays. Immunoprecipitations were performed using nuclear extracts or fractions from the Superose 6HR column. ChIP assays were performed as described (Takahashi *et al*, 2000). A detailed description of the methods is found in the supplementary information online.

Post-transcriptional gene silencing of WSTF. HeLa cells were transfected with ~1 μg pSUPER (Brummelkamp *et al*, 2002) encoding either control shRNA encoding a scrambled WSTF sequence or shRNA against WSTF (GAACAGGAAGTTGCTGAGC). At 30 h after transfection using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA), RNA was prepared, reverse transcribed into complementary DNA using random primers and amplified by PCR using primers specific for 45S rRNA, 18S rRNA, 28 rRNA, $tRNA_{lys}$ and IL-6 mRNA. PCR products were quantified with Quantity One according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

***In vitro* transcription assays.** Transcription on naked DNA (pMrWT/*Nde*I, $pHrP_2$/*Eco*RI and $pMrT_2$) and on chromatin templates (Längst *et al*, 1997), and the abortive transcription initiation assay have been described (Philimonenko *et al*, 2004).

Antibodies. Antibodies to WSTF were from Cell Signaling (Beverly, MA, USA), SNF2h from Abcam (Cambridge, UK), rabbit IgGs from Dianova (Hamburg, Germany) and UBF, RPA194 and TFIIIC220 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against actin and NM1 (Percipalle *et al*, 2001;

Fomproix & Percipalle, 2004), TAF$_I$110 and RPA116 have been described (Seither & Grummt, 1996; Heix *et al*, 1997). The antibodies to fibrillarin (72B9) and SNF2h/ISWI were gifts from M. Pollard (La Jolla, CA, USA) and P. Wade (Atlanta). The human autoimmune serum S57299 against Pol I was obtained from a scleroderma patient.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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